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Previews

Designing Effective Hybrid Toxins

A study of a well-designed androgen-mustard conjugate provides evidence supporting a novel mechanism for its selective toxicity in androgen-receptor-positive cancer cells [1]. This represents a solid step forward on the path toward effective hybrid toxins for targeted cancer therapy.

The concept of hybrid toxins emerged many years ago as researchers sought to tame the non-specific toxicity of standard antitumor agents by conjugating them to targeting moieties. The hope for these hybrid toxins was that the targeting element would direct them or their action more selectively to the tumor and thereby maintain their effectiveness at this site while sparing the morbidity resulting from non-tumor organ toxicity.

Hybrid toxins have taken many forms—antibodies conjugated to peptide toxins or enzymes to activate antitumor prodrugs; small-molecule ligands for cell-surface or intracellular receptors conjugated to cytotoxic chemotherapeutics—and work on them has spanned many decades. Nevertheless, progress in fulfilling their promise has been slow in coming. Moreover, the history of hybrid-toxin development is filled with reports that claim effectiveness based on a targeting mechanism that is either quantitatively untenable or is poorly supported by the details of the study [2].

For example, Estramustine (Figure 1A), a estradiol-nitrogen mustard conjugate [3], was claimed to act by binding to steroid receptors in prostate tumors and thereby delivering the toxic mustard selectively to these sites [4]. Although it is used to treat prostate cancer, Estramustine does not bind to steroid receptors, and its antimitotic action appears to arise through a mechanism very different from that originally claimed. It binds to microtubule-associated proteins [5], an interaction that does not even require the reactive chlorines of the mustard.

In this issue of *Chemistry & Biology*, Marquis et al. [1] (from the laboratories of Essigmann and McCroy) describe the synthesis and evaluation of newly designed androgen-mustard conjugates (Figure 1B). Not only have they uncovered very interesting bioactivity of their key conjugate, but their study also exemplifies many of the elements of careful design and of appropriate control experiments that are needed to advance the promise of hybrid toxins in a rational manner [2, 6].

The authors of this report have a different perspective on how a hybrid toxin—specifically, a steroid-mustard conjugate—might bring about a selective toxic effect in a receptor-positive cancer cell [1]. In contrast to the traditional view that might be termed a "bind-then-alkylate" mechanism (see Figure 1C, pathway A), they envision actions by an alternative "alkylate-then-bind" mechanism (Figure 1C, pathway B). The selective toxic effect through pathway A is presumed to result from selective uptake and retention of the steroid mustard conjugate by receptor-positive cells and thereby lead to a higher level of DNA alkylation in these cells. By contrast, selective toxicity through pathway B is not envisioned to arise from selective alkylation, but rather from an impairment of DNA repair in receptor-positive cells (and possibly from other mechanisms). The binding of steroid receptors to steroids that are tethered to sites of DNA alkylation is presumed to sterically block access of DNA-repair enzymes to the lesion and thereby trigger apoptotic pathways in the receptor-positive cells, but not in the receptor-negative cells in which these lesions could be repaired. A secondary result of receptor binding to these DNA adducts might be the sequestration of the receptor away from important regulatory sites needed to support cell proliferation or survival. Distinguishing between the two principal mechanisms—pathway A versus pathway B—is a challenge; in fact, with a properly designed hybrid toxin, both pathways might be active.

In the work described in this report [1], the authors have built upon their careful prior studies with indole-based estrogen-mustard conjugates [7–9] to construct a new hybrid toxin, an androgen-mustard conjugate (Figure 1B). In their design, a chlorambucil nitrogen mustard moiety is connected through a 15-atom linker to the high-affinity steroidal androgen; attachment to the steroid is at the 11β position, known to be a substituent-tolerant site. As an appropriate "toxin-inactive control" compound, they have prepared a steroid conjugate with a nonreactive mustard analog in which the reactive chlorines in the chlorambucil unit are replaced with methoxyl groups. As a "nonbinding toxin control" compound, they use chlorambucil itself.

The investigators then demonstrated that their androgen conjugates, both the hybrid toxin and toxin-inactive control compound, retain good affinity (Kd $\approx$ 1 nM) for the androgen receptor (AR), ca. one-fifth that of the in vivo active androgen 5α-dihydrotestosterone (DHT). However, the AR binding affinity of the steroid-mustard conjugate covalently attached to a model oligonucleotide is 20-fold lower than that of the free hybrid toxin. Even with this reduction, they find that the hybrid toxin has very favorable activities in cell culture and tumor xenograft models.

In AR-positive prostate cancer (LNCaP) cells, the androgen-mustard causes cell-cycle arrest and induces apoptosis. Chlorambucil itself and the toxin-inactive control steroid both cause cell-cycle arrest, but not apoptosis. Detailed studies of important regulators of the cell cycle and apoptosis, and other controls, support the unique apoptotic activity of the androgen-mustard conjugate. Particularly striking is the strong growth-suppressive effect of the androgen-mustard conjugate on LNCaP tumors grown as xenografts in nude mice, where a 90% reduction in tumor growth rate is sustained for many weeks. Notably, though, the compound is cytostatic, not cytotoxic.

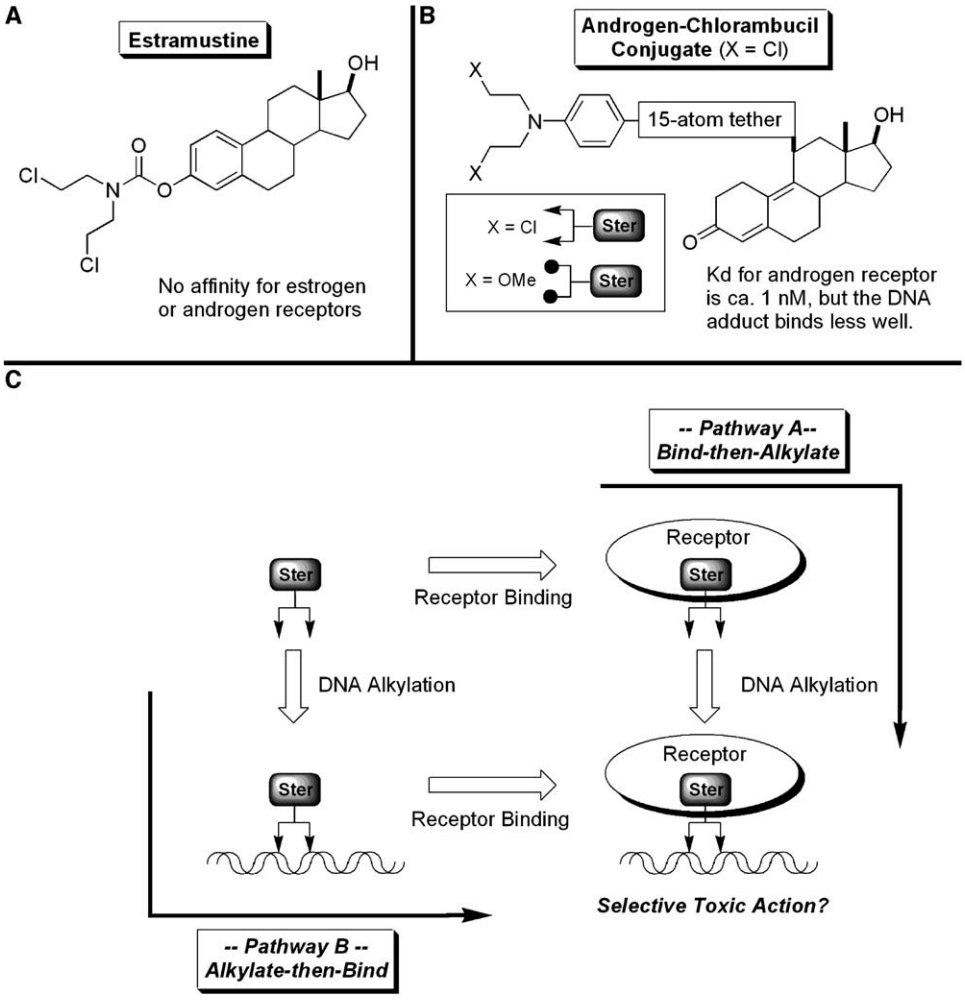

Figure 1. Steroid-Mustard Conjugates and Alternative Pathways Proposed for Their Action

Estramustine (A) is an active agent in prostate cancer treatment; it is proposed to act via pathway A, but it does not bind to steroid receptors. The androgen-chlorambucil conjugate (B), the topic of this commentary [1], is selectively cytotoxic to androgen receptor-positive cells and cytostatic in xenografts models, and it binds to the androgen receptor; its selective action is proposed to arise via pathway B. Selective toxicity toward receptor-positive cells by pathway A ("bind-then-alkylate") is proposed to involve selective uptake of the steroid-mustard conjugate in receptor positive cells and selective DNA alkylation in these cells. By contrast, selective toxicity by pathway B ("alkylate-then-bind") is proposed to involve steric occlusion of DNA lesion repair by receptor binding to the tethered steroid; actions resulting from the sequestration of the receptor from important promoter sites might also occur.

That a well-designed androgen hybrid toxin is more active than are the toxin control and binding control compounds in AR-positive cells and that it shows substantial antitumor activity in an animal model of AR-positive cancer are very interesting findings, but is this hybrid toxin working by a receptor-mediated "bind-then-alkylate" or an "alkylate-then-bind" pathway (Figure 1C), or is it working by a receptor-independent pathway altogether? Although distinguishing between the two receptor-mediated pathways will be difficult, it is worth considering how additional controls might more firmly establish that the activities observed for the androgen hybrid toxin are, indeed, receptor mediated [2, 6].

It would be helpful to have a more ideal nonbinding toxin control compound than chlorambucil itself. Tethering chlorambucil to a steroid-like molecule with no AR binding affinity would give an agent having cell uptake and pharmacokinetic properties more like that of the androgen-chlorambucil conjugate. If it were still less effective than the hybrid toxin, it would demonstrate that the unique activities of the androgen-mustard derived from its AR binding, not from differences in cell-uptake rates or biodistribution.

The role of AR in mediating the effects of the androgen-chlorambucil hybrid toxin could be substantiated by further controlled studies in cells. Comparative toxicity studies in AR-positive versus AR-negative cell lines might be informative, although inherent differences in the response of different cell lines to cytotoxic agents might make interpretation of the results difficult. Comparing the activity of the hybrid toxin in the same AR-positive cells in which the AR has been knocked down with siRNA could be especially informative, as might studies in which nonreactive AR ligands are used to block the interaction of the androgen-chlorambucil hybrid toxin with AR [6]. Further refinement in the design of androgen-toxin conjugates could also be considered. In particular, if one could develop compounds

in which the androgen-tethered DNA adduct retained high affinity for the AR, then selectivity toxicity by an "alkylate-then-bind" mechanism becomes more likely.

Although additional mechanistic questions remain, the findings presented are both intriguing and promising. Considering the current limitations in the effectiveness of both hormonal and cytotoxic chemotherapy of prostate cancer, the results also represent a solid step toward fulfilling the promise of hybrid toxins as more selective cancer chemotherapeutic agents. Of equal importance, this study nicely illustrates the careful thought that needs to be given to the design of hybrid toxins and the careful controls that are required to interpret mechanistically their activities in cell and animal model systems. This work will serve both as a standard and an inspiration for future studies in this area.

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Selected Reading

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